



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/18, 9/00	A1	(11) International Publication Number: WO 98/19695 (43) International Publication Date: 14 May 1998 (14.05.98)
<p>(21) International Application Number: PCT/IB97/01398</p> <p>(22) International Filing Date: 5 November 1997 (05.11.97)</p> <p>(30) Priority Data: 08/746,021 5 November 1996 (05.11.96) US</p> <p>(71) Applicant: MODEX THERAPEUTIQUES [CH/CH]; 27, rue du Bergnon, CH-1005 Lausanne (CH).</p> <p>(72) Inventors: AEBISCHER, Patrick; 163, route de Lausanne, CH-1096 Villette (CH). DEGON, Nicole; Ancien Collège, CH-1521 Curtilles (CH). REGULIER, Etienne; 84A, avenue de Béthusy, CH-1012 Lausanne (CH). RINSCH, Christopher; 84A, avenue de Béthusy, CH-1012 Lausanne (CH).</p> <p>(74) Agent: KIRKER & CIE S.A.; 122, rue de Genève, P.O. Box 324, CH-1226 Thônex (CH).</p>		<p>(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>
<p>(54) Title: DEVICE AND METHOD FOR DELIVERY OF ERYTHROPOIETIN</p>		
<p>(57) Abstract</p> <p>A device and method for delivery of EPO to a patient using an implanted device that continuously releases EPO.</p>		

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DEVICE AND METHOD FOR DELIVERY OF ERYTHROPOIETIN

TECHNICAL FIELD OF THE INVENTION

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This invention relates to devices and methods for delivery of erythropoietin ("EPO"), preferably using encapsulated EPO-secreting cells.

BACKGROUND OF THE INVENTION

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Erythropoietin is an essential growth factor in the erythrocytic lineage. This glycoprotein hormone is secreted mainly in the kidney peritubular cells and in the fetal liver in response to hypoxia. Jelkman W, Physiol Reviews 72(2):449 (1992). In addition, cobalt chloride also induces Epo production. Goldwasser E. et al, Blood 13:55 (1958). Tissue hypoxia is the primary stimulus for production of erythropoietin which levels may rise to 10000 U/l plasma in severe anemia as compared to the normal value of 5-20 U/l.

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The injection of recombinant erythropoietin (EPO) is now widely used for long-term treatment of anemia associated with chronic renal failure, cancer and human immunodeficiency virus infections. Recombinant human erythropoietin (rhEPO) has revolutionised the treatment of anemia in patients on maintenance haemodialysis,

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abolishing the need for blood transfusions. Winearls CG et al., Lancet 2(8157):1175 (1986); Gimenez LF et al., Hematology/Oncology Clinics of North America 8(5):913 (1994).

5 The injection of rhEPO is widely used as a replacement therapy for anemic patients. In chronic renal failure, anemia results from a destruction of EPO-secreting cells. In chronic diseases, loss of EPO-secreting cells also occurs. Such diseases include inflammatory diseases (e.g., rheumatoid arthritis and cancers) or infectious diseases (e.g., AIDS).

10 Treatment is often performed as self-administered subcutaneous (s.c.) injection which is thought to be more effective than the intravenous (i.v.) route, although the bioavailability of rhEPO is low. Jensen JD et al., Eur J Clin Pharmacol 46:333 (1994); Stockenhuber F et al., Nephron 59:399 (1991.)

15 The ability to deliver this hormone by gene or cell therapy rather than by repeated injections could provide substantial clinical and economic benefits. In one embodiment we contemplate using genetically modified cells secreting high levels of human Epo.

20 Permanent *in vivo* delivery of EPO according to the devices and methods of this invention could provide great benefits in terms of cost and clinical procedures. The median weekly dose of EPO is about 150 U/kg, administered by one to three injections. The annual cost of treating a 70 kg man is about 10000 Swiss francs.

Implantation of polymer encapsulated EPO-secreting cells would abolish the need of repeated injections without affecting the biological efficacy of EPO in treatment of human hemoglobinopathies.

25 The cell therapy approach described here is expected to provide a convenient means for continuous delivery of EPO from a retrievable implant.

SUMMARY OF THE INVENTION

This invention provides a novel methods and devices for delivering EPO. In

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one embodiment, one or more tethered devices, each containing typically between 10^3 and 10^8 cells, preferably 10^5 to 10^7 cells, most preferably about 10^6 genetically modified cells, surrounded by a semipermeable membrane, are implanted in the patient providing for continuous release of EPO. The dosage delivered can be varied, and is
 5 sufficient to produce a therapeutic effect. Further, preferably the encapsulated cells express high levels of EPO, and are hypoxia responsive, increasing expression of EPO under hypoxic conditions.

The semipermeable membrane prevents immunologic rejection of the cells and interposes a physical, preferably virally impermeable, barrier between cells and
 10 host. Moreover, the device and the cells it contains may be retrieved from the patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Plasmid map of pPI-neo-DHFR-Epo vector containing the human EPO
 15 gene. Features of the vector include mouse PGK promoter (bp 14-523); chimeric intron (bp 588-720); T7 promoter (bp 765-783); EPO cDNA (bp 1077-1577); T3 promoter (bp 1796-1816); SV40 late polyadenylation signal (bp 1825-2046); phage f1 region (bp 2141-2596); SV40 enhancer and early promoter (bp 2660-3078); SV40 minimum origin of replication (bp 2976-3041); coding region of neomycin
 20 phosphotransferase (bp 3123-3917); synthetic polyadenylation signal (bp 3981-4029); SV40 promoter (bp 4050-4408); mutated DHFR gene Fnu4II1 (bp 4409-5059); HBV-3' UTR (bp 5060-5647); β -lactamase coding region (bp 6427-7287).

Fig. 2: Plasmid map of pCI-neo-DHFR-EPO vector containing the human EPO
 25 gene. Features of the vector include cytomegalovirus immediate-early enhancer (bp 1-659); cytomegalovirus immediate-early promoter (bp 669-750); chimeric intron (bp 890-1022); T7 promoter (bp 1067-1085); EPO cDNA (bp 1379-1879); T3 promoter (bp 2097-2118); SV40 late polyadenylation signal (bp 2127-2348); phage f1 region (bp 2443-2898); SV40 enhancer and early promoter (bp 2962-3380); SV40 minimum

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origin of replication (bp 3278-3343); coding region of neomycin phosphotransferase (bp 3425-4219); synthetic polyadenylation signal (bp 4283-4331); SV40 promoter (bp 4352-4710); mutated DHFR gene Fnu4H1 (bp 4711-5361); HBV-3' UTR (bp 5362-5949); β -lactamase coding region (bp 6729-7589).

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Fig. 3: In vitro production of EPO by engineered myoblasts under various oxygen concentrations. EPO secretion is expressed in UI/ 10^6 cells/day.

Fig. 4: In vitro production of EPO by encapsulated engineered myoblasts. Three
10 capsules were exposed to a regime of normoxic conditions for 24 hours, hypoxic conditions for 24 hours, and then returned to normoxic conditions for 24 hours. EPO secretion is expressed in UI/ 10^6 cells/day.

Fig. 5: In vivo regulation of EPO production by encapsulated engineered
15 myoblasts in hypoxic mice. Mice were implanted with encapsulated EPO secreting cells and exposed to normoxic conditions or hypoxic conditions. The graph shows the correlation between capsule EPO secretion (UI/24 hours) and serum EPO concentration (mUI/ml).

20 Fig. 6: In vivo regulation of human EPO production by encapsulated engineered myoblasts implanted into rats. The serum EPO levels (mUI/ml) are given two rats, over a 96 hour time frame, from prior to implant to post-explant. This figure shows the control experiment, in which the rats were exposed to normoxic oxygen conditions for the duration of the experiment.

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Fig. 7: In vivo regulation of human EPO production by encapsulated engineered myoblasts implanted into rats. The serum EPO levels (mUI/ml) are given two rats, over a 96 hour time frame, from prior to implant to post-explant. This figure shows

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the treatment experiment, in which the rats were exposed to normoxic oxygen conditions for 24 hours, then hypoxic oxygen conditions for 24 hours, and returned to normoxic conditions for 24 hours. The capsule was then explanted.

5 DETAILED DESCRIPTION OF THE INVENTION

The devices and methods of this invention permit long-term delivery of high doses of rhEPO by implantation of polymer encapsulated cells genetically modified to secrete EPO into the serum. The provision of an encapsulating membrane of appropriate molecular weight cutoff shields the transplanted cells from the host
10 immune system, thereby allowing the transplantation of cell lines across species. The dosage provided is sufficient to achieve a therapeutic effect in the patient. In one embodiment, living cells are encapsulated in one or more semipermeable polymer capsules and surgically inserted (under local anesthesia) into the patient.

This technique provides several advantages over other delivery routes:

- 15 (1) Drug can be delivered to the patient continuously thereby reducing fluctuations in dosage;
- (2) If cells are viable for long periods of time (such as six months), patients will be inconvenienced only twice a year instead of three times weekly or every day with subcutaneous injections;
- 20 (3) Since viable cells continuously produce newly synthesized product, these cells should have advantages over pump delivery of drug stores, where drug is continuously degraded but not continuously replenished.

In one embodiment we contemplate cellular immunoisolation of Epo-producing allogenic or xenogenic cells surrounded by a selective membrane barrier for
25 the treatment of human hemoglobinopathies. The implantation of these genetically modified encapsulated cell-lines is a convenient method to achieve permanent *in vivo* delivery of the hormone.

Cellular immunoisolation of EPO-producing allogenic or xenogenic cells

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provides a safe and simple therapy for patients suffering from anemia due to, e.g., chronic renal failure and beta-thalassemia. Capsules implanted subcutaneously can be removed and/or replaced easily. Second, a long-term correction of anemia by constant delivery of EPO would greatly enhance the patient's quality of life and decrease the cost of treatments.

The circulating form of human EPO is a 165 amino acid glycoprotein with an apparent molecular weight of about 30,000 daltons. The encapsulated cells of this invention produce EPO. Such cells may naturally produce EPO, or may be genetically engineered to do so. Such genetic engineering may involve transfection of cells with a vector containing a nucleotide sequence encoding EPO, preferably human EPO.

Alternatively, such genetic engineering may involve DNA targeting by homologous recombination to result in high level of expression an endogenous EPO gene. Such techniques are well known in the art. See WO 93/09222, incorporated herein by reference.

The human gene encoding EPO consists of five exons and five introns. The preferred EPO used in the present invention is the nucleotide and amino acid sequence encoding the human protein, such as described in U.S. patents 4,703,008 and 5,547,933 which are incorporated herein by reference. Modified, truncated, mutein and active fragment forms of EPO are well known and are contemplated by this invention. See, e.g., 5,457,089; 5,166,322; 4,835,260; and 5,106,954, all of which are specifically incorporated herein by reference.

In a preferred embodiment, full length recombinant human EPO (rhEPO) is used.

In vivo production of rat EPO in mice or rats have reported a consequent increase in hematocrit level. Naffakh N et al., Proc Natl Acad Sci USA 92:3194 (1995); Osborne WRA et al., Proc Natl Acad Sci USA 92:8055 (1995). Our approach, involving encapsulation of cells in a polymeric film with known physicochemical characteristics of molecular-weight-cut-off and chemical composition

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helps control the interaction between the host and the transplanted tissue. This effective immunoisolation of the transplanted tissue may render possible xenogenic transplantation in a broad spectrum of patients.

5 A gene of interest (i.e., a gene that encodes a suitable biologically active molecule, e.g., EPO) can be inserted into a cloning site of a suitable expression vector by using standard techniques. It will be appreciated that more than one gene may be inserted into a suitable expression vector. These techniques are well known to those skilled in the art.

10 The expression vector containing the gene of interest may then be used to transfect the desired cell line. Standard transfection techniques such as calcium phosphate co-precipitation, DEAE-dextran transfection or electroporation may be utilized. Commercially available mammalian transfection kits may be purchased from e.g., Stratagene.

15 A wide variety of host/expression vector combinations may be used to express the gene encoding EPO, or other biologically active molecule of interest.

Suitable promoters include, for example, the early and late promoters of SV40 or adenovirus and other known non-retroviral promoters capable of controlling gene expression.

20 Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., pUC, pBlueScript™ plasmids from E. coli including pBR322, pCR1, pMB9, pUC, pBlueScript™ and their derivatives.

25 Expression vectors containing the geneticin (G418) or hygromycin drug selection genes (Southern, P.J., In Vitro, 18, p. 315 (1981). Southern, P.J. et al., J. Mol. Appl. Genet., 1, p. 327 (1982)) are also useful. These vectors can employ a variety of different enhancer/promoter regions to drive the expression of both a biologic gene of interest (e.g., NGF) and/or a gene conferring resistance to selection with toxin such

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as G418 or hygromycin B. The G418 resistance gene codes for aminoglycoside phosphotransferase (APH) which enzymatically inactivates G418 (100-500 g/l) added to the culture medium. Only those cells expressing the APH gene will survive drug selection usually resulting in the expression of the second biologic gene as well. The
5 hygromycin B phosphotransferase (HBH) gene codes for an enzyme which specifically modifies hygromycin toxin and inactivates it. Genes cotransfected with or contained on the same plasmid as the hygromycin B phosphotransferase gene will be preferentially expressed in the presence of hygromycin B at 50-200 g/ml concentrations.

10 A variety of different mammalian promoters can be employed to direct the expression of the genes for G418 and hygromycin B and/or the biologic gene of interest. Examples of expression vectors that can be employed are the commercially available pRC/CMV, pRC/RSV, and pCDNA1NEO (InVitrogen).

In one embodiment, the pNUT expression vector is used. Baetge et al.,
15 PNAS, 83, pp. 5454-58 (1986). In addition, the pNUT expression vector can be modified such that the DHFR coding sequence is replaced by the coding sequence for G418 or hygromycin drug resistance. The SV40 promoter within the pNUT expression vector can also be replaced with any suitable constitutively expressed mammalian promoter, such as those discussed above.

20 In a preferred embodiment, we contemplate insertion of the human Epo cDNA into high expression vectors containing housekeeping gene promoter such as CMV (Cytomegalovirus), PGK-I (Phospho-glycerate kinase-I), or MT-I (Metallothionein). We most prefer the PGK-1 promoter. The sequence of the PGK-1 promoter is known. Adra et al., Gene, 60, pp. 65-74 (1987).

25 Regulation of expression may be achieved using hypoxia-sensitive regulatory elements. Previous studies have shown that promoter and enhancer elements in the 5' and 3' flanking region of the human erythropoietin gene could promote the increase of the EPO production under hypoxic conditions. The human

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EPO hypoxia inducible enhancer region comprises a tripartite structure which begins 115 nucleotides 3' to the EPO adenylation site. Beck et al., J Biol Chem 266(24):15563 (1991); Beck et al., Blood 82(3):70 (1993); Blanchard KL et al., Mol Cell Biol 12(12):5373 (1992); Goldberg MA et al., Proc Natl Acad Sci USA 84:7972 (1987); Madan A et al., Proc Natl Acad Sci USA 90:3928 (1993).

Alternatively, other suitable hypoxia responsive elements may be used to generate an EPO-producing cell in which the production of EPO is regulated. In a preferred embodiment we contemplate use of cells transfected with at least one hypoxia responsive element from the 5' flanking sequence of the phosphoglycerate kinase I gene. The PGK-1 hypoxia responsive element is an 18 mer element within the PGK-1 promoter sequence, and has the following sequence:

5' GTCGTGCAGGACGTGACA 3'

This element has sequence and protein binding similarities to the hypoxia inducible factor 1 binding domain within the EPO 3' enhancer.

Insertion of more than one copy of this PGK-derived 18 mer is contemplated to provide greater hypoxia responsiveness. Preferably between two to ten copies are inserted 5' to the PGK promoter (or other suitable promoter) in the transfected cells. In a specific embodiment we contemplate insertion of a tandem repeat of the 18 mer, having the following sequence:

5' GTCGTGCAGGACGTGACA CTCGC GTCGTGCAGGACGTGACA 3'

Both allogeneic and xenogeneic cells may be used. Use of a cell line of xenogeneic origin provides an additional advantage since the transplanted cells will be rejected by the host immune system in the event of device breakage.

These cells are surrounded with a permselective membrane which permits the diffusion of small molecules such as nutrients and trophic factors into and out of the polymer envelope, while excluding larger molecules of the immune system (antibodies, complement, etc.).

A wide variety of cells may be used. These include well known, publicly

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available immortalized cell lines as well as dividing primary cell cultures. Examples of suitable publicly available cell lines include, chinese hamster ovary (CHO), mouse fibroblast (L-M), NIH Swiss mouse embryo (NIH/3T3), African green monkey cell lines (including COS-1, COS-7, BSC-1, BSC-40, BMT-10 and Vero), rat adrenal pheochromocytoma (PC12 and PC12A), AT3, rat glial tumor (C6), astrocytes and other fibroblast cell lines. Primary cells that may be used include, EGF-responsive neurospheres, bFGF-responsive neural progenitor stem cells derived from the CNS of mammals (Richards et al., PNAS 89, pp. 8591-8595 (1992); Ray et al., PNAS 90, pp. 3602-3606 (1993)), primary fibroblasts, Schwann cells, astrocytes, β -TC cells, Hep-G2 cells, oligodendrocytes and their precursors, myoblasts (including C2C12 cells) and the like.

Cell lines offer several advantages including unlimited availability, the possibility of rapid screening *in vitro* for the presence of pathogens from which cell banks are established, and the suitability for stable gene transfer using non-viral-based recombinant DNA techniques. One preferred cell line chosen for the gene transfer technique are baby hamster kidney (BHK) cells. Other preferred cell lines are myoblasts, including C2C12 cells, most preferably C2C12 cells.

A particular advantage to using xenogeneic over allogeneic cells is that in the unlikely event of membrane failure, the xenogeneic cells are more likely to be targeted for destruction by the immune system when compared to allogeneic cells. Furthermore, xenogeneic sources are easy to obtain and their use precludes the necessity for the use of human tissue which is difficult to obtain and fraught with societal and ethical considerations. In addition, human tissue may contain adventitious agents that are more readily transmitted to the transplantation recipient. Finally, use of xenogeneic tissue and cell lines for transplantation in humans removes the risks associated with the handling and processing of human tissue.

Increased expression can be achieved by increasing or amplifying the copy number of the transgene encoding EPO or other suitable biologically active

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molecule(s), using amplification methods well known in the art. Such amplification methods include, e.g., DHFR amplification (see, e.g., Kaufman et al., United States patent 4,470,461) or glutamine synthetase ("GS") amplification (see, e.g., United States patent 5,122,464, and European published application EP 338,841).

5 EPO levels may be monitored either by ELISA or by bioassay (Naffakh et al., Proc. Natl. Acad. Sci. USA, 92, pp. 3194-98 (1995)).

The methods of this invention may be used in combination with other therapies. Likewise, other molecules may be co-delivered to the patient, e.g., immunosuppressive molecules such as CR-1.

10 Co-delivery can be accomplished in a number of ways. Cells may be transfected with separate constructs containing the genes encoding the described molecules. Alternatively, cells may be transfected with a single construct containing two or more genes.

Multiple gene expression from a single transcript is preferred over
15 expression from multiple transcription units. One approach for achieving expression of multiple genes from a single eukaryotic transcript takes advantage of sequences in picorna viral mRNAs known as internal ribosome entry sites ("IRES"). These sites function to facilitate protein translation from sequences located downstream from the first AUG of the mRNA.

20 Macejak and Sarnow reported that the 5' untranslated sequence of the immunoglobulin heavy chain binding protein (BiP, also known as CRP 78, the glucose-regulated protein of molecular weight 78,000) mRNA can directly confer internal ribosome binding to an mRNA in mammalian cells, in a 5'-cap independent manner, indicating that translation initiation by an internal ribosome binding
25 mechanism is used by this cellular mRNA. Macejak, Nature, 353, pp. 90-94 (1991).

WO 94/24870 refers to use of more than two IRES for translation initiation from a single transcript, as well as to use of multiple copies of the same IRES in a single construct.

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Various modified IRES sequences are known. See, e.g., Mountford and Smith, Trends Genet., 11, pp. 179-84 (1995); Dirks et al., Gene, 128, pp. 247-49 (1993); Martinez-Salas et al., J. Virology, 67, pp. 3748-55 (1993) and Mountford et al., Proc. Natl. Acad. Sci. USA, 91, pp. 4303-07 (1994). Use of these modified
5 sequences is also contemplated in this invention.

Also contemplated is encapsulation of two or more separately transfected cells or cell lines, each secreting one of the desired molecules.

This invention also contemplates use of different cell types during the course of the treatment regime. For example, a patient may be implanted with a capsule device containing a first cell type (e.g., hEPO-secreting BHK cells). If after
10 time, the patient develops an immune response to that cell type, the capsule can be retrieved, or explanted, and a second capsule can be implanted containing a second cell type (e.g., C₂C₁₂ cells). In this manner, continuous provision of the therapeutic molecule is possible, even if the patient develops an immune response to one of the
15 encapsulated cell types.

Encapsulation hinders elements of the immune system from entering the capsule, thereby protecting the encapsulated cells from immune destruction. This technology increases the diversity of cell types that can be employed in therapy. The semipermeable nature of the capsule membrane also permits the molecule of interest
20 to easily diffuse from the capsule into the surrounding host tissue. This technique prevents the inherent risk of tumor formation and allows the use of unmatched human or even animal tissue, without immunosuppression of the recipient. Moreover, the implant may be retrieved if necessary or desired. It is both undesirable and expensive to maintain a patient in an immunosuppressed state for a substantial period of time.
25 Such retrievability may be essential in many clinical situations.

Numerous encapsulation devices are known, having various outer surface morphologies and other mechanical and structural characteristics. Capsules have been categorized as Type 1 (T1), Type 2 (T2), type 1/2 (T1/2) or Type 4 (T4) depending

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on their outer surface morphology. Such membranes are described, e.g., in Lacy et al., Science, 254, pp. 1782-84 (1991), Dionne et al., PCT/US92/03327 and Baetge, WO 95/05452.

As used herein "a biocompatible capsule" means that the capsule, upon
5 implantation in a host mammal, does not elicit a detrimental host response sufficient to result in the rejection of the capsule or to render it inoperable, for example through degradation.

As used herein "an immunoisulatory capsule" means that the capsule upon
implantation into a mammalian host minimizes the deleterious effects of the host's
10 immune system on the cells within its core.

A variety of biocompatible immunoisulatory capsules are suitable for
delivery of molecules according to this invention. Such capsules will allow for the
passage of metabolites, nutrients and therapeutic substances while minimizing the
detrimental effects of the host immune system. Preferably the capsule of this
15 invention will be similar to those described in Aebischer et al., PCT publication WO 92/19195, incorporated herein by reference.

Useful biocompatible polymer capsules comprise (a) a core which contains
a cell or cells, either suspended in a liquid medium or immobilized within a hydrogel or
extracellular matrix components (or other suitable molecules), and (b) a surrounding or
20 peripheral region of permselective matrix or membrane (jacket) which does not contain
isolated cells, which is biocompatible, and which is sufficient to protect isolated cells
if present in the core from detrimental immunological attack.

The core of the polymer capsule is constructed to provide a suitable local
environment for the continued viability and function of the cells isolated therein.

25 Many transformed cells or cell lines are most advantageously isolated within a capsule having a liquid core. For example, cells can be isolated within a capsule whose core comprises a nutrient medium, optionally containing a liquid source of additional factors to sustain cell viability and function, such as fetal bovine or

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equine serum.

Suitably, the core may be composed of a matrix formed by a hydrogel which stabilizes the position of the cells in cell clumps. The term "hydrogel" herein refers to a three dimensional network of cross-linked hydrophilic polymers. The network is in the form of a gel, substantially composed of water, preferably but not limited to gels being greater than 90% water.

Compositions which form hydrogels fall into three classes. The first class carries a net negative charge (e.g., alginate). The second class carries a net positive charge (e.g., collagen and laminin). Examples of commercially available extracellular matrix components include Matrigel™ and Vitrogen™. Fibroblasts generally survive well in a positively charged matrix and are thus suitably enclosed in extracellular-matrix type hydrogels. The third class is net neutral in charge (e.g., highly crosslinked polyethylene oxide, or polyvinylalcohol). Any suitable matrix or spacer may be employed within the core, including precipitated chitosan, synthetic polymers and polymer blends, microcarriers and the like, depending upon the growth characteristics of the cells to be encapsulated.

Preferably, the capsules are immunoisulatory. To be immunoisulatory, the surrounding or peripheral region of the capsule should confer protection of the cells from the immune system of the host in whom the capsule is implanted, by preventing harmful substances of the host's body from entering the core of the vehicle, and by providing a physical barrier sufficient to prevent detrimental immunological contact between the isolated cells and the host's immune system. The thickness of this physical barrier can vary, but it will always be sufficiently thick to prevent direct contact between the cells and/or substances on either side of the barrier. The thickness of this region generally ranges between 5 and 200 microns; thicknesses of 10 to 100 microns are preferred, and thickness of 20 to 75 microns are particularly preferred. Types of immunological attack which can be prevented or minimized by the use of the instant vehicle include attack by macrophages, neutrophils, cellular

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immune responses (e.g. natural killer cells and antibody-dependent T cell-mediated cytotoxicity (ADCC), and humoral response (e.g., antibody-dependent, complement-mediated cytotoxicity).

Use of immunoisulatory capsules allows the implantation of xenogeneic cells or tissue, without a concomitant need to immunosuppress the recipient. Use of immunoisulatory capsules also allows use of unmatched cells (allografts). The type and vigor of an immune response to xenogeneic cells is expected to differ from the response encountered when syngeneic or allogeneic tissue is implanted into a recipient. This response may proceed primarily by cell-mediated, or by complement-mediated attack; the determining parameters in a particular case may be poorly understood. However, the exclusion of IgG from the core of the vehicle is not the touchstone of immunoprotection, because in most cases IgG alone is insufficient to produce cytotoxicity of the target cells or tissues. Using immunoisulatory macrocapsules, it is possible to deliver needed high molecular weight products or to provide metabolic functions pertaining to high molecular weight substances, provided that critical substances necessary to the mediation of immunological attack are excluded from the immunoisulatory capsule. These substances may comprise the complement attack complex component C3b, or they may comprise phagocytic or cytotoxic cells; the instant immunoisulatory capsule provides a protective barrier between these harmful substances and the isolated cells. Thus, an immunoisulatory capsule can be used for the delivery even from xenogeneic cells, products having a wide range of molecular sizes. Accordingly, for immunoisulatory capsules nominal molecular weight cutoff (MWCO) values between 50 - 2000 kD are contemplated. Preferably, the MWCO is between 50-700 kD. Most preferably, the MWCO is between 70-300 kD.

Various polymers and polymer blends can be used to manufacture the capsule jacket, including polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers, polyurethanes, polystyrenes,

polyamides, cellulose acetates, cellulose nitrates, polysulfones (including polyether sulfones), polyphosphazenes, polyacrylonitriles, poly(acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof.

The capsule can be any configuration appropriate for maintaining biological activity and providing access for delivery of the product or function, including for example, cylindrical, rectangular, disk-shaped, patch-shaped, ovoid, stellate, or spherical. Moreover, the capsule can be coiled or wrapped into a mesh-like or nested structure. If the capsule is to be retrieved after it is implanted, configurations which tend to lead to migration of the capsules from the site of implantation, such as spherical capsules small enough to travel in the recipient's blood vessels, are not preferred. Certain shapes, such as rectangles, patches, disks, cylinders, and flat sheets offer greater structural integrity and are preferable where retrieval is desired.

In one embodiment, the implantable capsule is of a sufficient size and durability for complete retrieval after implantation. Preferably the device has a tether that aids in retrieval. Such tethers are well known in the art. Such macrocapsules have a core of a preferable minimum volume of about 1 to 10 μl and depending upon use are easily fabricated to have a volume in excess of 100 μl .

In one embodiment the capsule will have an inner single ultrafiltration membrane with a permselective pore-size permeability range of 60-98% BSA rejection coefficient and 50-90% ovalbumin rejection coefficient.

In a hollow fiber configuration, the fiber will typically have an inside diameter of less than 1500 microns, preferably less than 300-600 microns. In either geometry, the hydraulic permeability will typically be in the range of 1-100 $\text{mls/min/M}^2/\text{mmHg}$, preferably in the range of 25 to 70 $\text{mls/min/M}^2/\text{mmHg}$. The glucose mass transfer coefficient of the capsule, defined, measured and calculated as described by Dionne et al., ASAIO Abstracts, p. 99 (1993), and Colton et al., The Kidney, eds., Brenner BM and Rector FC, pp. 2425-89 (1981) will be typically greater than 10^{-6} cm/sec , preferably greater than 10^{-4} cm/sec .

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T1 membranes may be formed by coextrusion of a polymer solution and coagulant solution through air before entering a quench bath. T2 membranes may be formed by coextruding the polymer, and coagulation solutions into humidified air or a mist and then into a bath. T4 membranes may be formed by coextrusion of the polymer and coagulant solutions directly into a coagulant bath, so that formation of the permselective membrane occurs on both outer and inner wall surfaces simultaneously. Methods of making biocompatible, immunoisulatory semipermeable hollow fiber membranes are disclosed in United States patents 5,284,761 and 5,158,881, herein incorporated by reference.

10 T1/2 membranes may be formed using similar methods used to form T2 membranes. However, the mist or humidity at the coextrusion port may be controlled according to known methods to produce the desired outer surface morphology. Alternatively, the nozzle distance from a quench bath may be varied, according to routine methods. Further, if coextrusion is used to cast the membrane, the absolute and/or relative flow rates of polymer and coagulant may be adjusted to achieve the desired outer wall surface morphology. Finally, the polymer and coagulant solution compositions and temperatures can be varied to achieve the desired outer surface wall morphology. See WO 95/0542.

20 Any suitable method of sealing the capsules may be used, including the employment of polymer adhesives and/or crimping, knotting and heat sealing. These sealing techniques are known in the art. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced. Subsequent to filling, the capsule is sealed. Such a method is described in copending United States application 25 Serial No. 08/082,407, herein incorporated by reference.

In a preferred embodiment, the capsule is formed from a polyether sulfone hollow fiber, such as those described in United States Patent Nos. 4,976,859 and 4,968,733, herein incorporated by reference. Such capsules jackets may be

permselective, or may be microporous. In other embodiments we contemplate using microcapsules for cellular delivery. See, e.g., United States patents 4,352,883; 4,353,888; and 5,084,350, herein incorporated by reference. Likewise, we also contemplate polymer rods (e.g., EVA rods) for delivery of EPO.

5 The methods and devices of this invention are intended for use in a mammalian host, recipient, patient, subject or individual, preferably a primate, most preferably a human.

 A number of different implantation sites are contemplated for the devices and methods of this invention. These implantation sites include subcutaneous,
10 intravenous, intramuscular, preferably subcutaneous.

 The actual dosage can be varied by any suitable method known in the art, including, e.g., by implanting a fewer or greater number of capsules. If the cells are encapsulated in macrocapsules, we prefer implanting between one and ten capsules. The dosage should be sufficient to produce a therapeutic effect. The cell loading
15 density may also be varied over a wide range. If macrocapsules are used, typically between 10^3 and 10^8 cells are encapsulated per capsule, preferably 10^5 to 10^7 cells are encapsulated, most preferably about 10^6 cells.

 The cells, methods and devices of this invention may be useful in the treatment of human hemoglobinopathies. The clinical applications of EPO are rapidly
20 expanding.. Life-long treatment with very high doses is now under evaluation for hemoglobinopathies. Rodgers GP et al., New England Journal of Medicine 328:73 (1993). In particular, the devices and methods of this invention may be used in the treatment of disease states associated with deficient levels of EPO.

 Deficient levels of EPO production have been associated with numerous
25 forms of anemia, including anemia resulting from renal failure and end-stage renal disease, anemias of chronic infection, auto-immune diseases, anemia of prematurity, anemia of hypothyroidism, and anemia of malnutrition.

 For renal insufficiency, approximately 3000 to 10000 UI/week is currently

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administered by subcutaneous injection. For β -thalassemia, approximately 150,000 UI/day are administered. Using the devices and methods of this invention, delivery of such therapeutically effective amounts of EPO can routinely be achieved. Further, continuous capsular delivery may permit lowering the dosage requirement due to
5 higher bioavailability of the cell-produced EPO.

In one embodiment of this invention, we provide hypoxia responsive cells that produce up to between 644 -750 UI EPO/ 10^6 cells/day (produced under normoxic conditions). At about this level of production, to deliver between 3000 - 10,000 UI/week, a single macrocapsule containing about 10^6 cells would be sufficient to
10 deliver a therapeutic dosage. Likewise, to deliver up to 150,000 UI/day, e.g., two devices each containing about 10^8 cells would be sufficient. And, as noted above, a lower dosage may be required using the devices and methods of this invention, rather than traditional delivery methods.

Animal models are available for pre-clinical assessment. A mouse model of
15 beta-thalassemia caused by the deletion of the mouse beta-major globin gene has been described. Skow LC et al., Cell 34:1043 (1983). That model has been shown to be a reasonable model for studying specific therapies for human beta-thalassemia. Leroy-Viard K et al., Blood 78(6):1596 (1991); Villeval J-L et al., Blood 84(3):928 (1994). In these mice, repeated injections of EPO increase expression of the B-minor
20 globin, as well as the hemoglobin level, and improve the erythrocyte phenotype of beta-thalassemic mice -- i.e., it reduces the amount of unpaired and insoluble a-hemoglobin chains and the membrane protein defects, which characterize both murine and human beta-thalassemic erythrocytes.

Brunner et al., Kidney International 36:969 (1989); Brunner FP, Clinical
25 Nephrology 25:148 (1986) reref to a second model. They reported pharmacological studies in Wistar rats with markedly reduced renal mass. Typically, the left renal pedicle of the rats is carefully dissected through a midline abdominal incision. Then two or three upper branches of the left renal artery is tied off. One week later, the

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right kidney is removed after ligating the right renal pedicle from a flank incision. After few weeks, the rats are placed in metabolic cages.

Finally, a large animal model (normal and uremic sheep) has also been described. Eschbach JW et al., Kidney International 18:72 (1980); Mladenovic J et al., J Lab Clin Med 105(6):65 (1980); Widness JA et al., Journal of Pharmacology and Experimental Therapeutics 261(3):977 (1992); Eschbach JW et al., J Clin Invest 74:434 (1984). A model of chronic renal failure was created by two-stage, subtotal nephrectomy. Sheep are maintained in hemodialysis and stable uremia is created. The sheep is a good model because of two advantages: (1) vascular access can be maintained for a prolonged period of time free of infection, and (2) by selecting animals for hemoglobin phenotype, it is possible to take advantage of an unusual hematopoietic property of certain ruminants, the appearance of a structurally distinct hemoglobin, hemoglobin C, in response to erythropoietin stimulation.

15

EXAMPLES

Example 1: Construction of C2C12/EPO Cell Line

We constructed a C2C12 cell line secreting rhEPO. The cell line was tested in various in vitro and in vivo models.

20

Plasmid construction:

The plasmid pCI-Neo was purchased from Promega Corporation (Madison, WI, USA). This plasmid was modified as follows. A mutated dihydrofolate reductase gene (DHFR) under the control of the SV40 promoter as well as the Hepatitis virus B 3' untranslated region (HBV-3' UTR) were isolated from a modified pNUT vector (Baetge et al, Proc. Natl. Acad. sci. USA, 83, pp. 5454-58 (1986) digested with PvuII. The isolated fragment was subcloned into the pCI-Neo vector digested by *Bam*HI and blunted by filling in the protruding ends with T4 polymerase resulting in pCI-Neo-DHFR plasmid.

30

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The mouse phosphoglycerate kinase 1 (PGK-1) promoter was excised from pCI-PGK-GIP-R/IRES/GLP-1-R vector (kindly provided by B. Thorens, Lausanne, Switzerland) by *Bgl*II/*Eco*RI digestion and cloned into the *Bgl*II/*Eco*RI isolated pCI-Neo-DHFR backbone giving rise to pPI-Neo-DHFR vector.

- 5 The mouse MT-1 promoter was removed from a modified pNUT vector (Baetge et al., supra) by PCR, then digested with *Bgl*II/*Pvu*II and cloned into the *Bgl*II/*Eco*RI isolated pCI-Neo backbone resulting in the pMI-Neo vector. The mutated dihydrofolate reductase gene (DHFR) under the control of the SV40 promoter as well as the Hepatitis virus B 3' untranslated region (HBV-3' UTR) were
10 isolated from the RP3224D plasmid digested with *Bam*HI and cloned into *Bam*HI digested pMI-Neo vector resulting in pMI-neo-DHFR vector.

- The plasmid pBluescript II SK+ (Stratagene, CA, USA) containing the cDNA for human erythropoietin, kindly provided by Dr. Y. Beuzard (Laboratory of Experimental Gene Therapy, Paris, France), was digested by *Eco*RI/*Not*I. The
15 isolated fragment was then cloned into the three above described vectors. The integrity of the final constructs pCI-neo-DHFR-Epo (map shown in Figure 2), pMI-neo-DHFR-Epo and pPI-neo-DHFR-Epo (map shown in Figure 1) was confirmed by restriction analysis and sequencing.

20 *Cell culture and transfection of C₂C₁₂ cells:*

- Mouse C₂C₁₂ myoblasts derived from leg skeletal muscle of an adult C3H mouse were obtained from American Type Culture Collection (ATCC; CRL 1777, Rockville, MD). C₂C₁₂ cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2mM L-glutamine, 4.5 g/l glucose,
25 100U/ml penicillin, 100U/ml streptomycin. The cells were transfected by calcium phosphate precipitation (Mammalian transfection kit, Stratagene) with plasmids pCI-neo-DHFR-Epo, pMI-neo-DHFR-Epo and pPI-neo-DHFR-Epo.

 After 2 weeks of incubation with 0.8 mg/ml G418 cells were incubated with

increasing concentrations of methotrexate (1-200 μ M) over a 6 weeks period thus amplifying the number of copies of integrated plasmids. Stability of transgene expression was achieved by 6 alternating weeks of cell incubation with either 0.8 mg/ml G418 or 200 μ M MTX. Clones were obtained by limiting dilution.

5

Measurement of medium rhEPO concentrations:

C_2C_{12} cells were plated in growth medium at density of 1×10^5 cells per well in 6-well culture dishes (Costar). Differentiated encapsulated cells were placed in PC-1 medium, a serum free medium containing human recombinant protein (Hycor Biomedical Inc., CA). Conditioned media were obtained by incubating the C_2C_{12} cells encapsulated or not in 1ml of fresh medium for 1 hour. The samples were stored at -20°C until determination of rhEPO concentrations using an enzyme linked immunosorbent assay (ELISA) system (Quantikine IVD, R&D Systems, Minneapolis, MN).

Transfected cells were assayed for EPO production. Cells transfected with pCI-neo-DHFR-Epo produced (average secretion of pool) 10 UI/ 10^6 cells/day. Cells transfected with pMI-neo-DHFR-Epo produced (average secretion of pool) 60 UI/ 10^6 cells/day. Finally, pooled cells (or clones isolated by limiting dilution) transfected with pPI-neo-DHFR-Epo produced EPO as reported in the Table below.

20

huEpo secretion of pPI-neo-DHFR-huEpo-transfected C_2C_{12} cells

25	Pool	150 IU/ 10^6 cells/24h	1.2 μ g/ 10^6 cells/24h
	Pool/10 #5	305 IU/ 10^6 cells/24h	2.3 μ g/ 10^6 cells/24h
	Pool/10 #6	354 IU/ 10^6 cells/24h	2.7 μ g/ 10^6 cells/24h
	Pool/10 #10	436 IU/ 10^6 cells/24h	3.4 μ g/ 10^6 cells/24h
30	Clone #1	373 IU/ 10^6 cells/24h	2.9 μ g/ 10^6 cells/24h
	Clone #2	750 IU/ 10^6 cells/24h	5.8 μ g/ 10^6 cells/24h
	Clone #3	644 IU/ 10^6 cells/24h	5.0 μ g/ 10^6 cells/24h
	Clone #5	111 IU/ 10^6 cells/24h	0.9 μ g/ 10^6 cells/24h
	Clone #6	199 IU/ 10^6 cells/24h	1.5 μ g/ 10^6 cells/24h

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	Clone #7	640 IU/10 ⁶ cells/24h	4.9µg/10 ⁶ cells/24h
	Clone #8	458 IU/10 ⁶ cells/24h	3.5µg/10 ⁶ cells/24h
	Clone #9	417 IU/10 ⁶ cells/24h	3.2µg/10 ⁶ cells/24h
	Clone #10	339 IU/10 ⁶ cells/24h	2.6µg/10 ⁶ cells/24h
5	Clone #11	318 IU/10 ⁶ cells/24h	2.4µg/10 ⁶ cells/24h
	Clone #13	446 IU/10 ⁶ cells/24h	3.4µg/10 ⁶ cells/24h
	Clone #14	423 IU/10 ⁶ cells/24h	3.2µg/10 ⁶ cells/24h

After freezing, clone 3 produced about 4 µg/10⁶ cells/day.

10 After removal of drug selection for one month, no significant difference was detected in expression of rhEPO in transfected cells (data not shown), confirming the stability of transgene expression in myoblasts. The functional activity of the produced EPO was confirmed by incubation of culture supernatants with EPO-dependent murine cell line DaE7.

15 pPI-neo-DHFR-huEpo-transfected cells from pool/10 above (producing about 150 IU/10⁶ cells/24h) were evaluated for hypoxia responsiveness. Unencapsulated cells were cultured in Petri dishes on a thin film that is permeable to O₂ (Petriperm). The Petri dishes were exposed to a constant stream of O₂ at concentrations of 0, 10, 40, 159.6 (normoxic) or 722 mm Hg oxygen. As the
20 data in Figure 3 shows, there was hypoxic induction of EPO production -- under 10 mm Hg oxygen, this induction was 3-fold the normoxic level of EPO production.

Example 2: Cell Encapsulation

Control and transfected C₂C₁₂ cells were harvested and diluted in order to
25 obtain a final cell suspension of 1x10⁵ cells/µl. For experiments in which a matrix was used in the capsule core, the final cell density was 100,000 cells/µl in 1.75 mg/ml Zyderm (Collagène S.A., Lausanne, Switzerland). For experiments where no matrix was used, i.e., a liquid core in the capsule, the final cell suspension was also 100,000 cells/µl.

30 The cell suspension was then injected into microporous polyethersulfone

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hollow fibers (OD: 550 μ m, ID:350 μ m)(Akzo Nobel Faser AG, Wuppertal, Germany). These fibers are described in United States patents 4,976,859 and 4,968,733, herein incorporated by reference.

One centimeter long capsules were made by cutting them to appropriate lengths and heat sealing the ends. Each capsule contains approximately 2×10^5 cells at the time of encapsulation. Capsules were placed during 3 days in DMEM medium containing 2% FCS at 37 °C, 5% CO₂ for cell differentiation. Capsules were subsequently tested by ELISA assay and transfected in PC-1 medium for *in vitro* studies or implanted in mice for *in vivo* follow up biological effect.

The cells of Example 1, encapsulated with a matrix core as described above, were assayed *in vitro* for hypoxic induction of EPO release. The data is shown in Figure 4. A total of three capsules was used in this experiment. Each capsule was exposed to normoxic oxygen conditions (159.6 mm Hg oxygen) for a period of 24 hours and EPO production measured (shown as release 1 in Figure 4). The capsule was then exposed to hypoxic conditions (10 mm Hg oxygen) (159.6 mm Hg oxygen) for a period of 24 hours and EPO production measured (shown as release 2 in Figure 4). Finally, the capsule was then returned to normoxic conditions (159.6 mm Hg oxygen) for a period of 24 hours and EPO production measured (shown as release 3 in Figure 4). As the data in Figure 4 demonstrate, there is a between 5-8.5 fold increase in EPO production under hypoxic conditions. Further, EPO production by the encapsulated cells return to the normoxic level after return to normoxic conditions.

Example 3: Subcutaneous capsule implantation in adult rats and mice:

Adult female C57BL/6, DBA/2J and C3H mice obtained from Iffa Credo (Saint-Germain sur l'Abresle, France) were anesthetized by inhalation of methoxyflurane (Metofane). Animals were placed in a prone position for surgery. A subcutaneous dissection was performed with a vertical lumbar incision. Capsules were carefully placed at one or two sites under the cutaneous tissue. The incisions

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were closed with two layers resorbable sutures (Vicryl 6.0). Upon recovery, the mice were returned to the animal care facility where they had access to food and water *ad libitum*.

The procedure for implanting rats (Fisher adult males, about 220 grams)
5 was substantially the same as described above, except that the animals were anesthetized using pentobarbitol.

Hematocrit measurement:

Under general anesthesia, blood was drawn every two weeks from the tail
10 vein into heparinized capillary tubes. The hematocrit was the measured by the microhematocrit method.

Capsules histology:

Capsules kept *in vitro* and those retrieved from *in vivo* experiments were
15 fixed in 4% paraformaldehyde with 1% glutaraldehyde for three hours and dehydrated under alcohol cycle in preparation for glycol-methacrylate embedding (Leica). The capsules were cut at 6µm thickness and stained with cresyl violet and eosine.

Detection of antibodies anti-rhEPO in mice serum:

20 A specific immunoperoxidase procedure using the Vectastain ABC system (Vector) on nitrocellulose bands was developed. Serial dilutions of rhEPO (Eprex, Cilag Laboratories) from 100ng to 1ng were blotted on 0.45 µm nitrocellulose membranes. Non-specific binding sites were then blocked by incubating the bands with 5% milk in tris buffer saline (TBS) pH 7.6. Bands were covered overnight with
25 60 µm of mice sera or PBS for negative control. Biotinylated goat anti-mouse (IgG (H+L) (Vector) was incubated with the bands followed by addition of the preformed Avidin Biotinylated horseradish peroxidase macromolecular Complex (ABC) (Vector). Finally signal detection was achieved using the diaminobenzidine

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tetrahydrochloride (DAB) chromogen (Pierce) which draw brown spots on the bands. After each procedure step 3-5 washes in phosphate buffer saline (PBS) was performed.

We encapsulated the cells of Example 1 according to the encapsulation protocol of Example 2, and implanted capsules in mice as described above. In some capsules, the cells were immobilized in a matrix core, in others the cells were in a liquid core. Implanted mice were exposed to either a normoxic environment (21 % oxygen) or hypoxic conditions (7 % oxygen) for 24 hours. After this time, blood was taken from the animals for EPO assay. The data is shown in Figure 5. Mouse serum EPO level is plotted as a function of capsular secretion of EPO. As the Figure shows, there is a strong correlation between capsular EPO release and serum EPO level.

In further experiments with mice implanted as described above, we monitored in vivo regulation of erythropoietin production under normoxic and hypoxic conditions, as reported in the following table:

15

In-Vivo Regulation of Encapsulated C₂C₁₂ Cells
Expressing Human Erythropoietin

	Mouse	Normoxia Capsule (UI/24hrs)	Serum Capsule (mUI/ml)	Hypoxia Capsule (UI/24hrs)	Serum (mUI/ml)	Induction
20	1	3.3	56.8	3.2	95.8	1.69
	2	7.0	72.4	7.1	230.8	3.19
25	3	2.3	33.0	2.2	43.1	1.31
	4	3.7	74.3	3.8	100.8	1.36
	5	6.7	105.9	7.5	210.4	1.99
	6	8.9	113.5	9.1	211.8	1.87
30	Average	5.3	76	5.5	148.8	1.90

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We encapsulated the cells of Example 1 according to the encapsulation protocol of Example 2, and implanted capsules in rats according to the protocol described above. A total of four rats were used in this study (two control; two treatment). The serum EPO level in each rat was assayed before capsule implantation.

5 The control rats were implanted with EPO producing capsules and exposed to normoxic conditions for 72 hours. The capsules were then explanted. As Figure 6 shows, after implant, the serum EPO level rose, and then returned to its pre-implant level after the device was explanted.

10 In the treatment group, rats were assayed for serum EPO levels before implant. Rats were then exposed to normoxic conditions for 24 hours after implant, then exposed to hypoxic conditions for 24 hours, returned to normoxic conditions for 24 hours, and finally the device was explanted. The results are shown in Figure 7. As that Figure shows, serum EPO levels increased upon device implant, increased further under hypoxic conditions, returned to the normoxic serum level when rat 4 was re-
15 exposed to normoxic conditions, and finally returned to the pre-implant value at device explant. Rat 3 showed the same trend as rat 4 initially, but died during the course of the experiment.

WE CLAIM:

1. A device for delivering erythropoietin to a mammal comprising:
a core comprising erythropoietin producing cells;
a jacket surrounding the core that provides a physical barrier
between the mammal and the core, the jacket having a permeability
such that erythropoietin can diffuse therethrough into the mammal.
2. The device of claim 1 wherein the cells are hypoxia responsive such that the
level of erythropoietin production increases under hypoxic conditions.
3. The device of claim 2 wherein the cells are genetically engineered with a
hypoxia response element selected from the group consisting of the EPO
response enhancer and promoter and the PGK-1 hypoxia responsive
element.
4. The device of claim 1 wherein the cells produce more than 500 UI
erythropoietin/ 10^6 cells/day.
5. The device of claim 1 wherein the cells are myoblasts.
6. The device of claim 1 wherein the device is a macrocapsule.
7. The device of claim 6 wherein the macrocapsule is shaped to form a hollow
fiber or a flat sheet.
8. The device of claim 6 wherein the jacket has a molecular weight cutoff
between 50 and 2000 kD.

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9. A method for delivering erythropoietin to a mammal comprising implanting at least one device according to any one of claims 1-8.
10. A method for treating human hemoglobinopathies comprising delivering a therapeutically effective amount of erythropoietin to a human comprising implanting at least one device according to any one of claims 1-8, said device producing a therapeutically effective amount of erythropoietin.
11. A cell that produces at least 500 UI erythropoietin/ 10^6 cells/day and that is hypoxia responsive such that the level of erythropoietin production increases under hypoxic conditions.
12. The cell of claim 11 wherein the cell has been transfected with the PGK-1 hypoxia response element.

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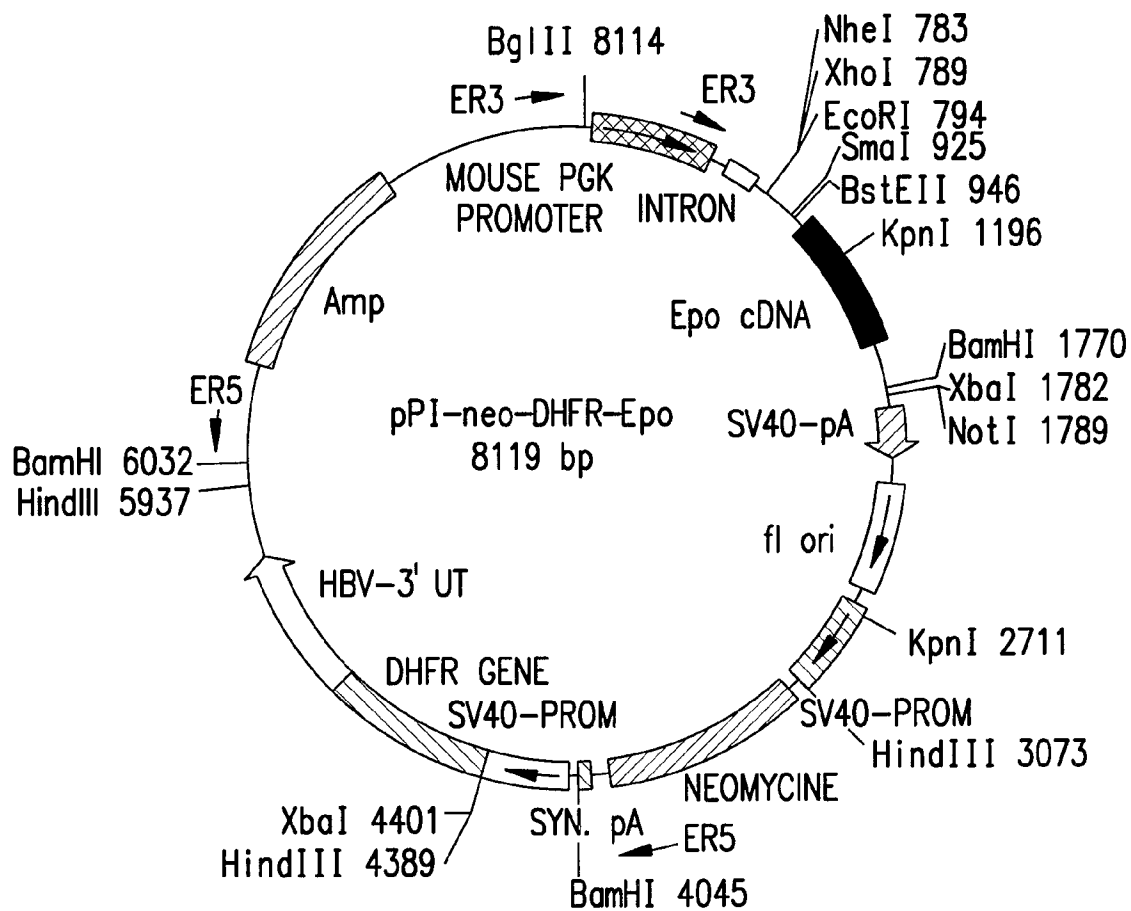


FIG.1

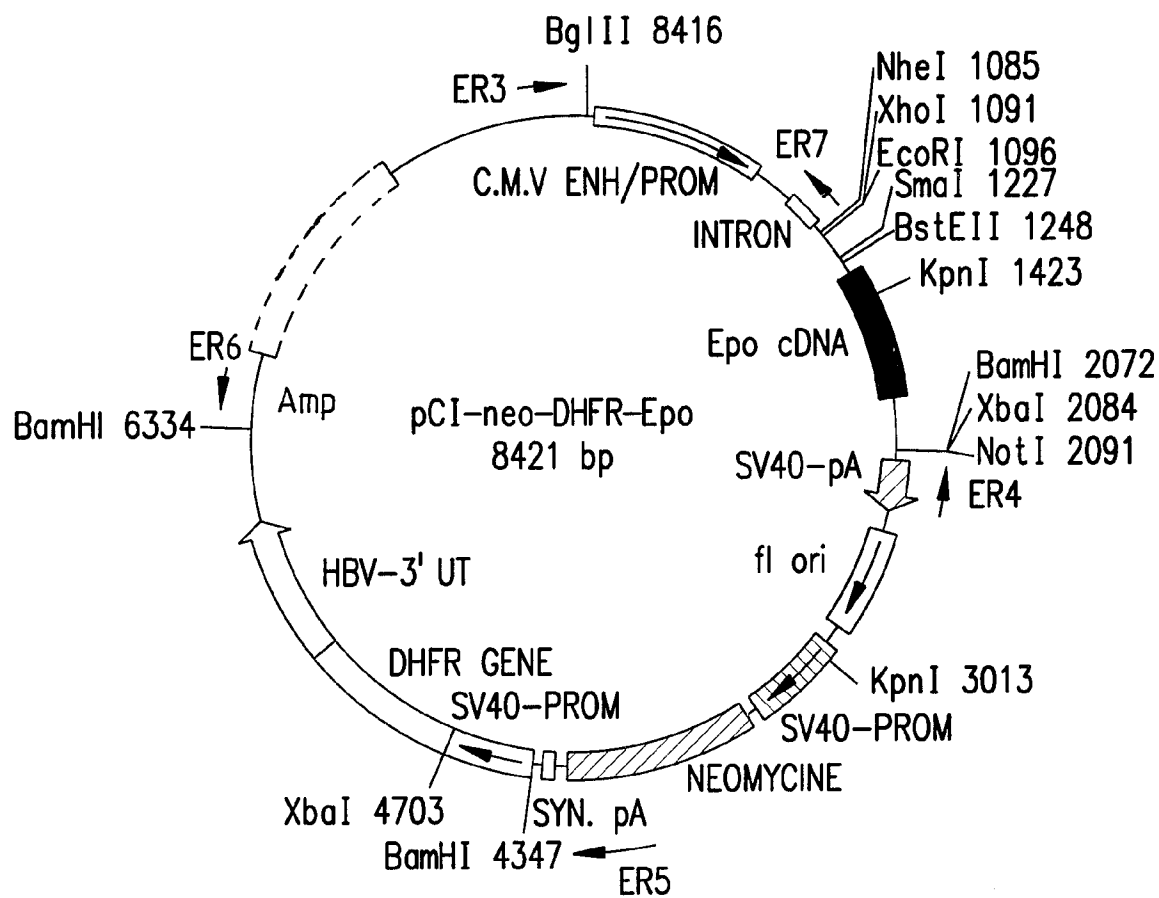


FIG. 2

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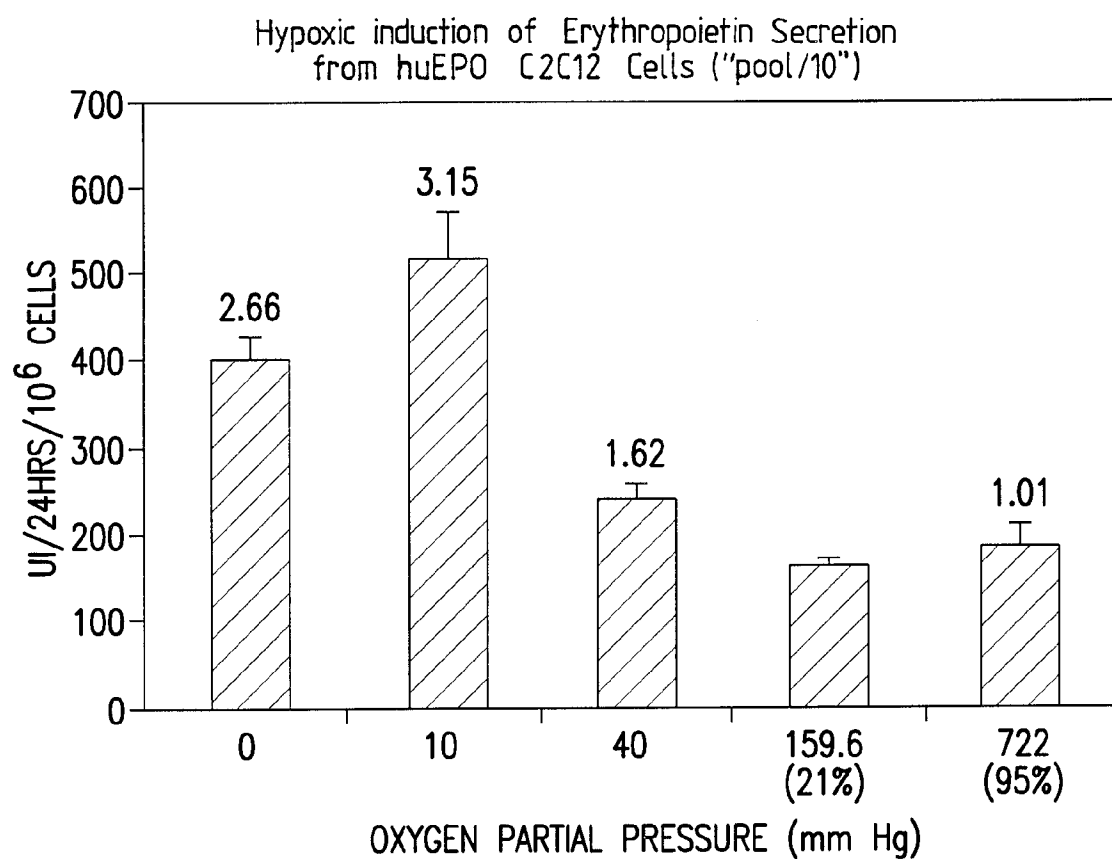


FIG.3

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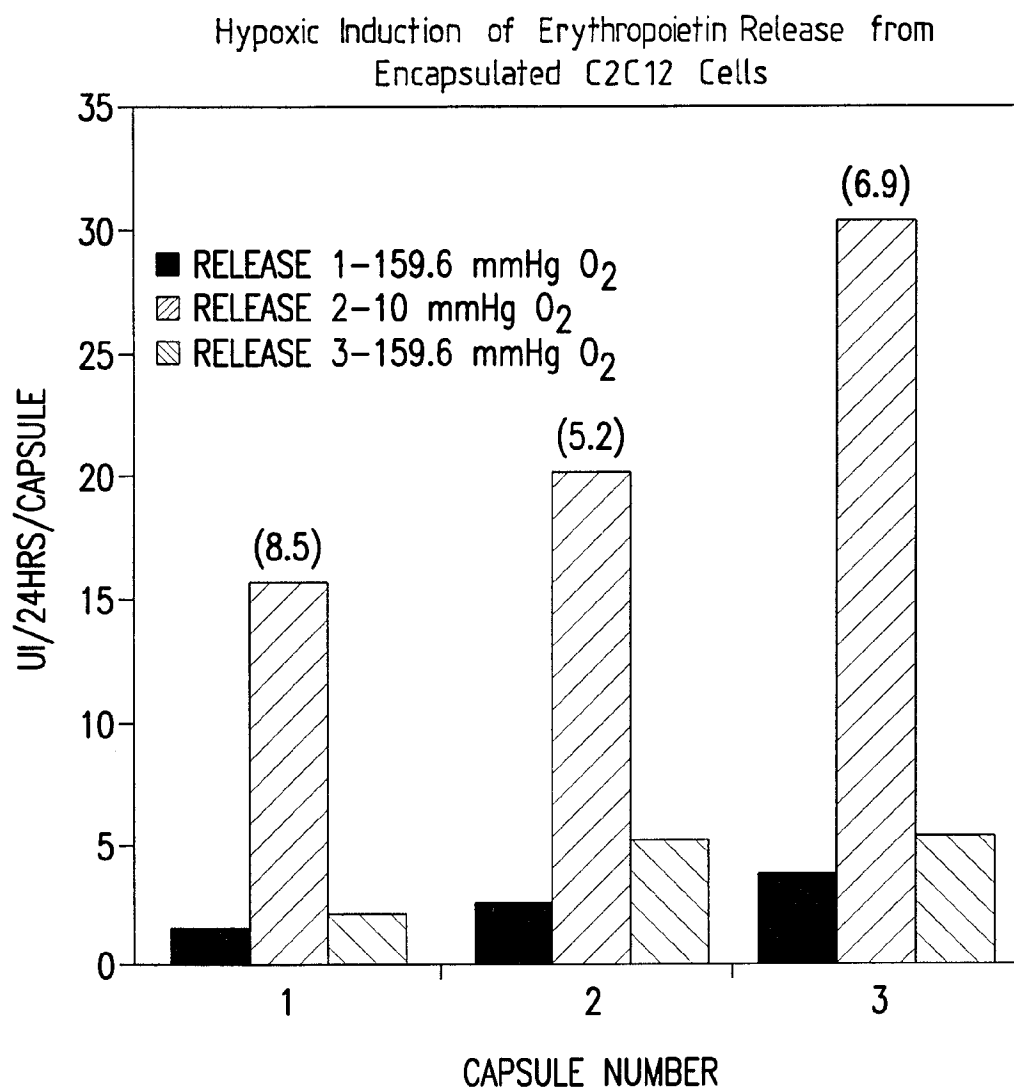


FIG.4

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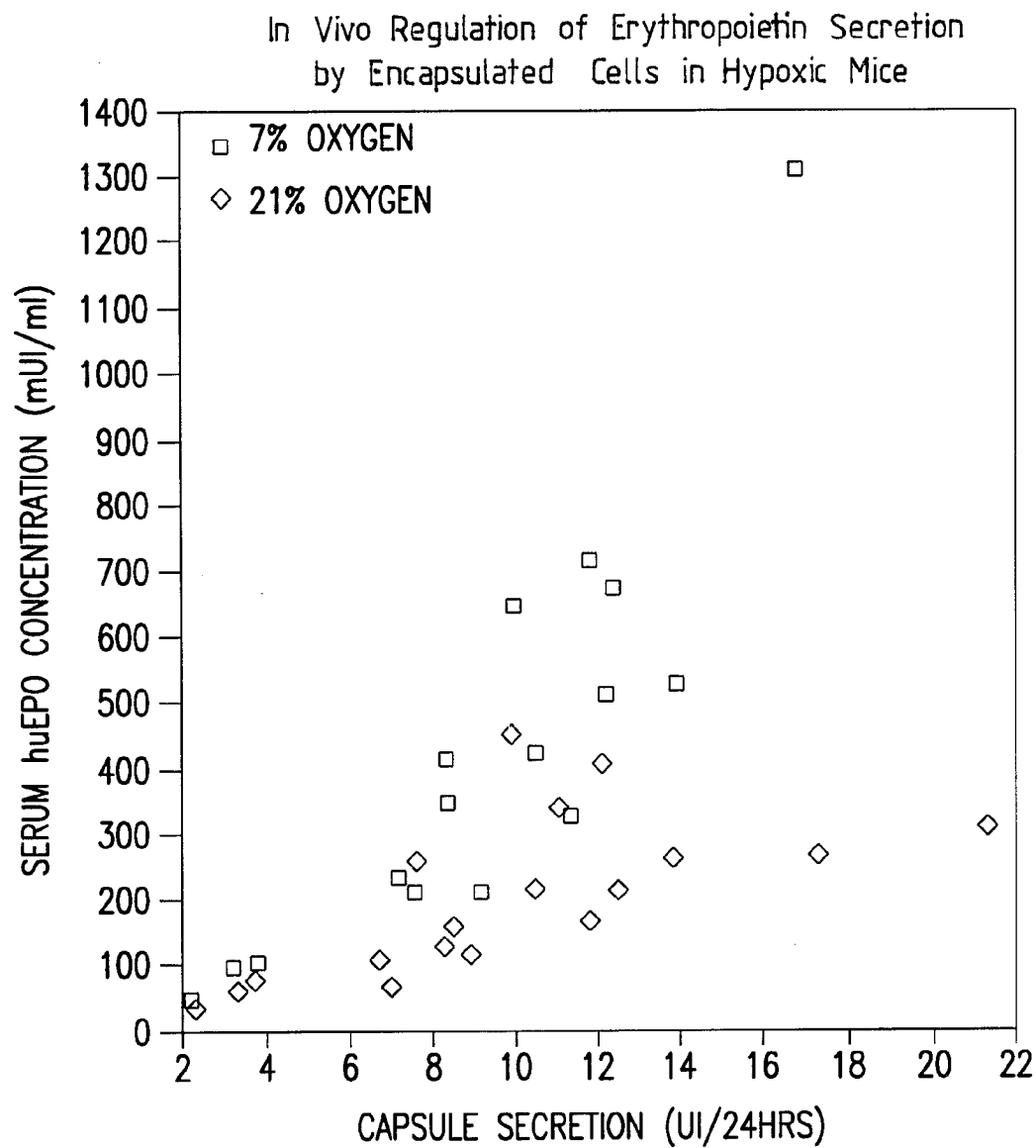


FIG.5

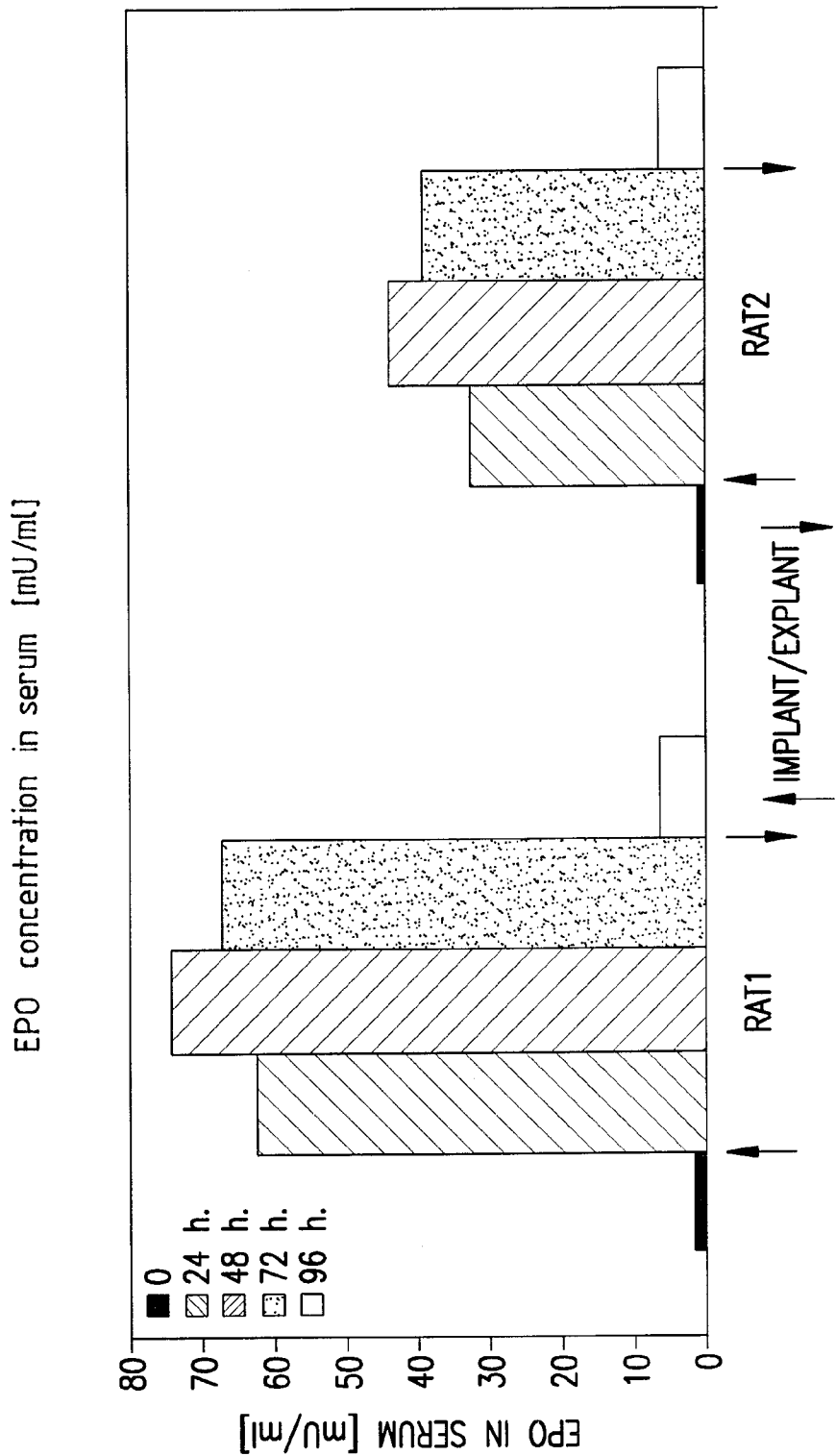


FIG.6

7/7

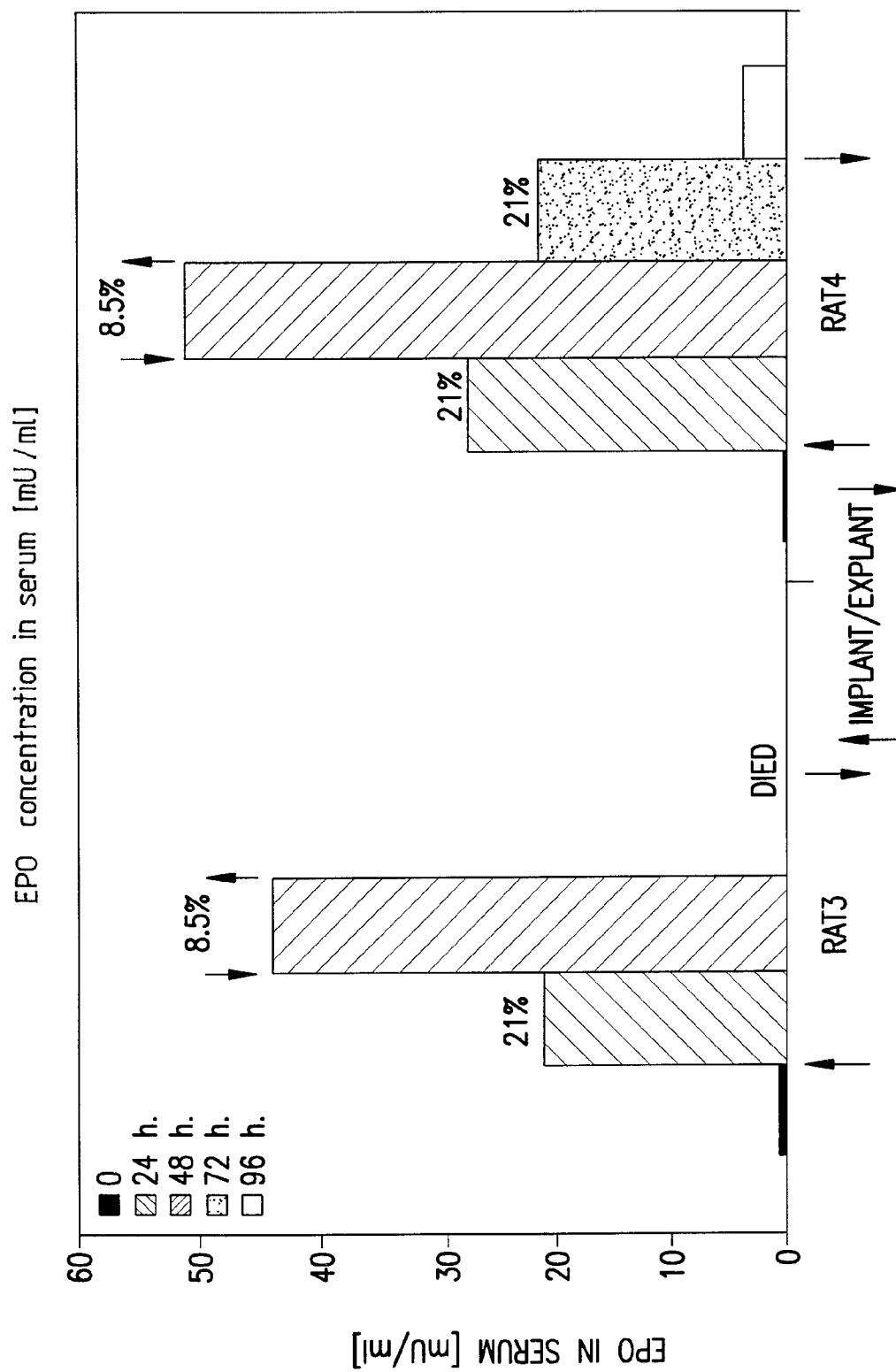


FIG.7

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 97/01398

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/18 A61K9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 19195 A (BROWN UNIVERSITY RESEARCH FOUNDATION) 12 November 1992 cited in the application see claims see page 36, line 27 see page 43, line 30 - line 32 ---	1-12
Y	WO 95 05452 A (CYTOTHERAPEUTICS) 23 February 1995 cited in the application see claims see page 12, line 27 ---	1-12
Y	WO 95 04521 A (W.L.GORE & ASSOCIATES) 16 February 1995 see claims see page 24, line 20 - line 30 see page 25, line 14 - line 33 --- -/--	1-12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

3 February 1998

Date of mailing of the international search report

13.02.98

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INTERNATIONAL SEARCH REPORT

Inter. .onal Application No

PCT/IB 97/01398

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	<p>WO 96 40871 A (CYTOTHERAPEUTICS) 19 December 1996 see claims 1-3,6,9-12,22 see page 14, line 17 - line 26 see page 19, line 7 - line 28 see page 20, line 4 - line 25 see page 21, line 28 -----</p>	1,2,4-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 97/ 01398

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9-10
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 9-10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 97/01398

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